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Two *cis* Elements Collaborate to Spatially Repress

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The expression pattern of many territory-specific genes in metazoan embryos is maintained by an active process of negative spatial regulation. However, the mechanism of this strategy of gene regulation is not well understood in any system. Here we show that reporter constructs containing regulatory sequence for the *SM30-α* gene of *Stronglyocentrotus purpuratus* are expressed in a pattern congruent with that of the endogenous *SM30* gene(s), largely as a result of active transcriptional repression in cell lineages in which the gene is not normally expressed. Chloramphenicol acetyl transferase assays of deletion constructs from the 2600-bp upstream region showed that repressive elements were present in the region from −1628 to −300. *In situ* hybridization analysis showed that the spatial fidelity of expression was severely compromised when the region from −1628 to −300 was deleted. Two highly repetitive sequence motifs, (G/A/C)CCCCT and (T/C)(T/A/C)CTTTT(T/A/C), are present in the −1628 to −300 region. Representatives of these elements were analyzed by gel mobility shift experiments and were found to interact specifically with proteins in crude nuclear extracts. When oligonucleotides containing either sequence element were co-injected with a correctly regulated reporter as potential competitors, the reporter was expressed in inappropriate cells. When composite oligonucleotides, containing both sequence elements, were fused to a misregulated reporter, the expression of the reporter in inappropriate cells was suppressed. Comparison of composite oligonucleotides with oligonucleotides containing single constituent elements show that both sequence elements are required for effective spatial regulation. Thus, both individual elements are required, but only a composite element containing both elements is sufficient to function as a tissue-specific repressive element.

INTRODUCTION

The sea urchin embryo is anatomically simple and lends itself well to studies of gene expression. At early stages it is composed of five gene expression territories which give rise to specific embryonic structures (reviewed in Davidson 1989, 1993). DNA microinjected into sea urchin zygotes is incorporated into nuclei during cleavage and undergoes rapid concatenation and amplification (Flytzanis *et al.*, 1985). Microinjected reporter constructs, containing regulatory sequences for various sea urchin genes and bacterial chloramphenicol acetyl transferase (CAT), are regulated in a spatially and temporally appropriate manner (Gan and Klein, 1990; Flytzanis *et al.*, 1987; Harlow *et al.*, 1989; Hough-Evans *et al.*, 1988; Katula *et al.*, 1987; Livant *et al.*, 1988; Niemeyer and Flytzanis, 1993; Sucov *et al.*, 1988). Studies of several regulatory regions have shown that coherent schedules of gene transcription are effected in sea urchin embryos by control regions that function as logic circuits, with separable spatial, temporal, and amplitude control elements (reviewed in Davidson, 1990; Coffman and Davidson, 1992). Maps of nuclear protein interaction sites for these upstream regions have shown that these regulatory elements are discrete modules which interact specifically with numerous sea urchin nuclear proteins (Theze *et al.*, 1990; reviewed in Coffman and Davidson, 1992). Genes encoding several of these proteins have been cloned (Coffman and Davidson, 1992) and some of the cloned genes appear to encode novel transcription factor peptide motifs. These studies show that gene transfer is an attractive approach to use for understanding regulatory region architecture and for identifying novel transcription factors.

The early spatial regulation of many eukaryotic genes is accomplished in part through tissue-specific repression of generally active promoters (reviewed in Herschbach and Johnson, 1993; Levine and Manley, 1989; Davidson, 1993). For these genes, which often mark the establishment of asymmetry between closely related cell types (e.g., Way *et*

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al., 1991; Winoto and Baltimore, 1989), it appears that negative control is as important as positive control in establishing the spatial boundries of gene transcription. In this present work, we initate our study of the spatial regulation of *SM30-α* reporter transcription. The *SM30* gene(s) of *Strongylocentrotus purpuratus* is expressed exclusively in the primary mesenchyme cell (PMC) lineage where it participates in the process of biomineralization and construction of the embryonic spicule (George *et al.*, 1991). Our results reveal that constructs express in a spatial pattern congruent with the endogenous *SM30* largely as a result of negative spatial regulation. We show that this repression is mediated through a specific and coordinated interaction of diffusible regulators with two DNA sequence elements.

MATERIALS AND METHODS

Construction of Reporter Constructs and Competitors

The 1.6CAT and +0.1CAT constructs were derived by replacing the *Hin*dIII–*Xba*I fragment of the multiple cloning site in the pCAT basic vector (Promega) with *Kpn*I–*Xba*I and *Xho*I–*Xba*I fragments from the *SM30-α* λgt11 genomic clone (Akasaka *et al.*, 1994). The 0.7 CAT and 0.3 CAT constructs were derived from the 2.6 CAT reporter by deleting the *Sal*I–*Eco*RV and *Sal*I–*Sfu*I(−300) fragments, respectively. An additional version of 0.3CAT was constructed by replacing the *Pst*I–*Xba*I fragment of the pCAT basic vector with the *Sfu*I–*Xba*I fragment from the λgt11 genomic clone. The 1.0CAT construct was derived by inserting the *Pvu*II–*Xba*I fragment from a pBluescript (SK+) (pBsk) subclone into the pCAT basic vector multiple cloning site (Promega). A second 0.3CAT construct was created by replacing the *Pst*I–*Xba*I fragment of the pCAT basic vector with the *Sfu*I–*Xba*I fragment from the 2.6CAT construct described above. At least two independent subclones of each construct, and several different plasmid preparations of each subclone, were used on different occasions and showed no significant differences in expression. Inserts were restriction mapped to confirm their identity and the 5′ and 3′ ends of the *SM30-α* sequence for each construct was sequenced from both directions using USB sequencing kit instructions. The −4.5 to +0.1, −1.6 to −1.0, and −0.3 to +0.1 competitors are the *Xba*I–*Xba*I, *Kpn*I–*Sac*I, and *Sfu*I(−300)–*Xba*I fragments from the λgt11 *SM30-α* genomic clone, respectively. All 0.3CAT fusion constructs were created by inserting the appropriate oligonucleotides into the *Pst*I site of the second 0.3CAT version described above.

Microinjection of Sea Urchin Zygotes, CAT Activity and Reporter Incorporation Assays, and CAT Activity Standardization

The collection of *S. purpuratus* gametes, microinjection of reporter construct DNA, and CAT enzyme activity assays were performed as described in McMahon *et al.* (1985). Quantitation was performed on a Molecular Dynamics phosphorimager. The lower limit of detection in CAT assays was equivalent to about 0.0016 units of bacterial CAT enzyme (Sigma) activity. Depending on the construct, an average batch of embryos contained about 0.0060 units of CAT enzyme activity. CAT activity was standardized to (divided by) the number of amplified reporter molecules per cell if the calculated number of replicated reporter molecules per nucleus was lower than 500 (presaturating levels, see below). Only samples with similar degrees of reporter replication were compared to one another. Carrier DNA for microinjection experiments was either linearized bluescript plasmid (pBsk) fragments, lacking the multiple cloning region, or genomic DNA partially digested with *Eco*RI to an average length of 10 kb. Although more substantial reporter amplification was obtained with the latter carrier, no qualitative or quantitative difference in relative reporter expression was observed between the two types of carrier.

Saturation and in Vivo Titration Experiments

Saturation experiments were conducted precisely as described by Livant *et al.* (1988). 2.6CAT reporter activity showed saturation kinetics; CAT activity approached a maximum when the reporter was amplified to 500–1000 copies per cell. *In vivo* competition of reporter expression was conducted as described in Livant *et al.* (1988) and Franks *et al.* (1990) except that competitor fragments were self-ligated to form low molecular weight concatenates (about 1–2 kb) which were not ligated to carrier DNA.

In Situ Hybridization

CAT mRNA localization was carried out as described in Ransick *et al.* (1993) with the following modifications: hybridization was carried out at 55°C using 100 ng/ml antisense riboprobe. Samples were treated with 50 μg/ml RNase A and 50 U/ml RNase T1 after riboprobe hybridization. Tissue assignment for stained cells was done, without knowledge of which experimental group was being examined, using 40× Nomarski optics. In interpreting *in situ* staining patterns, ectoderm and gut cell expression was indicated by reasonably contiguous, labeled cells in each territory. Mesenchyme cell expression was indicated by at least two normal sized and labeled cells occupying a position within the blastocoel.

Gel Mobility Shift Assays

In vitro DNA binding assays and affinity constant determinations were conducted using crude nuclear extract from 30 hr embryos as described in Calzone *et al.* (1988). Nonspecific competitor used was poly(dI–dC) and 2 μg of extract was used in each experiment.

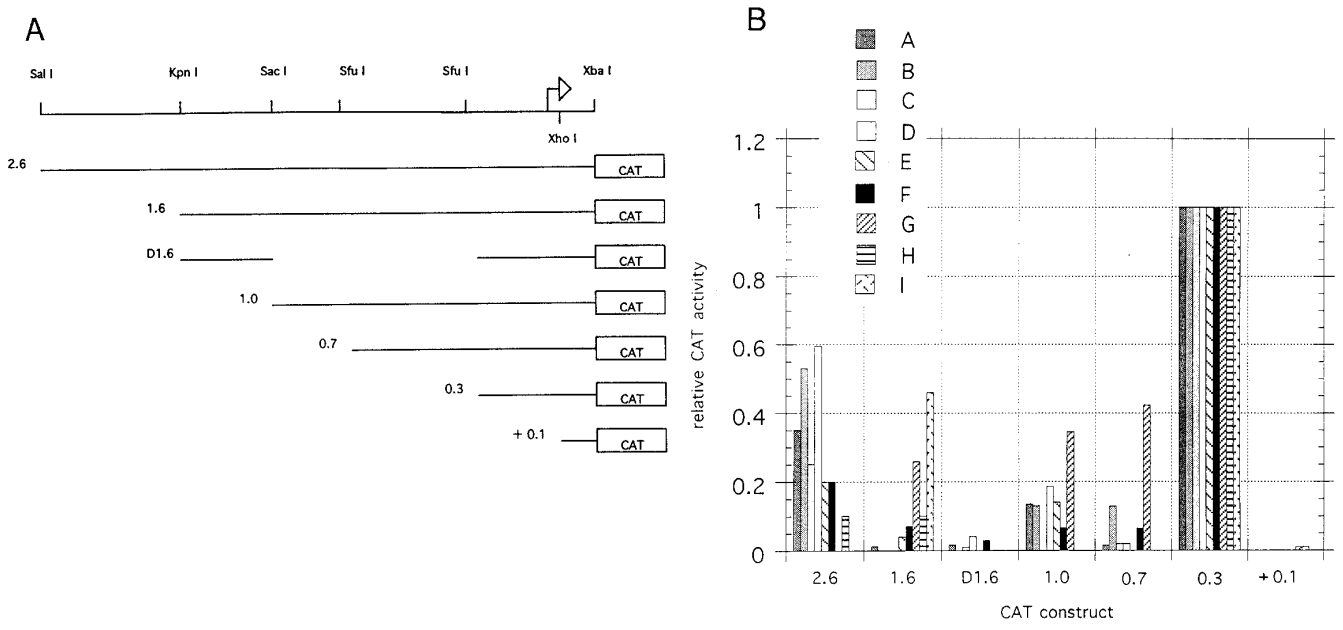


FIG. 1. Relative CAT activity of *SM30-α* deletion constructs. (A) Schematic depicting the 5′ and internal deletion constructs. Restriction sites used in their construction are listed above the line which represents the first 2.6 kb of *SM30-α* upstream sequence. The number to the left of each CAT construct identifies the construct. (B) Histogram of the relative CAT activity profile for the constructs shown in (A). Individual experiments are identified by capital letters and blocks (A–I) at the top of the histogram. The absence of a block for a given deletion construct in the histogram signifies that this construct was not among those assayed in that particular experiment. The CAT activity of all deletion constructs is shown relative to that of the 0.3CAT construct, which is arbitrarily set at 1 for each experiment.

RESULTS

Deletion Construct Analysis

As a first approach to the dissection of the 2.6-kb *SM30-α* regulatory region, we constructed various 5′ and internal deletion constructs of the 2.6CAT construct using convenient restriction sites (Fig. 1A). Groups of 60–100 injected embryos were collected and assayed for CAT enzyme activity and construct amplification. The results of several experiments were very similar; each showed a relative CAT activity profile in which 0.3CAT (containing the *SM30-α* sequence from −300 to +105) was significantly more active than the other, longer constructs (Fig. 1B). Since the CAT activity of the 1.6CAT, D1.6CAT, 1.0CAT, and 0.7CAT constructs is consistently less than that of the 0.3CAT construct, it is likely that there are multiple, perhaps functionally redundant, elements within the region spanning −1628 to −300 that have the ability to suppress expression directed by the −300 to +105 region.

To determine the spatial pattern of 1.6CAT and 0.3CAT transcription, we localized CAT mRNA using whole-mount *in situ* hybridization. Due to the mosaic nature of reporter construct incorporation (Hough-Evans *et al.*, 1988; Livant *et al.*, 1991), territories competent to express a given construct may not show expression in every cell of a given territory, nor in every competent territory of a given positively staining individual. Hence, the procedure requires the inspection of relatively large numbers of mosaic embryos to determine the territories of expression of a given construct.

The aboral ectoderm-specific *CyIIIa* CAT construct (characterized by Flytzanis *et al.*, 1987 and Hough-Evans *et al.*, 1988) was injected as a control and showed expression predominantly in patches of clonally related ectoderm cells of 48-hr gastrulae (Table 1). The *SM30-α* 2.6CAT construct, characterized by Akasaka *et al.* (1994), was also injected as a control. This construct shows significant CAT activity beginning at about 24 hr. *In situ* hybridization results show that this construct is expressed exclusively in PMCs of 24-hr blastulae (data not shown) and 48-hr gastrulae (Table 1; Akasaka *et al.*, 1994). The 1.6 CAT construct also shows significant CAT enzyme activity beginning about 24 hr (data not shown). *In situ* hybridization results show that the 1.6CAT reporter is expressed exclusively in the mesenchyme of 24-hr blastulae (average of 6 positive mesenchyme cells per positively staining embryo; Fig. 2A), 48-hr gastrulae (average of 2.8 positive mesenchyme cells per positively staining embryo; Figs. 2B and 2C; Table 1), and 96-hr plutei (average of about 2 mesenchyme cells per positively staining embryo; data not shown). It is presumed that later stages display fewer labeled cells because of instability of plasmid incorporation. The average number of PMCs expressing the *SM30-α* reporters at these three different stages, and the decline in this number with developmental time, is concor-

TABLE 1

In Situ Hybridization Experiments in 48-hr Gastrulae Harboring *SM30-α* Deletion Reporters: Number of Positive Embryos Observed per Territory

Construct	Number of positive embryos			Approximate number of embryos injected
	Ectoderm	Gut	Mesenchyme	
2.6CAT	1	8	273[a]	1550
1.6CAT	4	6	123[a]	1400
0.3CAT	6	101[a]	114[a]	1000
0.1CAT	0	0	0	600
CyIIIa CAT	37[a]	2	1	200
CAT	0	0	0	600
UN	0	0	0	5000
Average number of stained cells per positive embryo	8.0[a]	8.0[a]	2.8[a]	

[a] Data contributing to the determination of the average number of stained cells per territory, per positively stained embryo.

dant with expectations based on reports by others of reporter construct expression in the mesenchyme cells of blastula, gastrula, and prism stage sea urchin embryos (Sucov *et al.*, 1988; Gan and Klein, 1990; Hough-Evans *et al.*, 1990). Therefore we conclude that the 1.6CAT construct exhibits mesenchyme-specific regulation in *S. purpuratus* embryos.

The expression of 2.6CAT and 1.6CAT in PMCs of 24-hr blastulae was unexpected because this stage is slightly earlier than the start of endogenous *SM30* expression (George *et al.*, 1991). At this stage, PMCs are in the process of ingression into the blastocoel from the vegetal plate, and *in situ* hybridization shows that both 2.6CAT (Akasaka *et al.*, 1994) and 1.6CAT (Fig. 2A) are robustly expressed in these PMCs. 2.6CAT and 1.6CAT expression in PMCs still residing in the vegetal plate suggests that transcription of *SM30-α* is a function of lineage, not mesenchymal position.

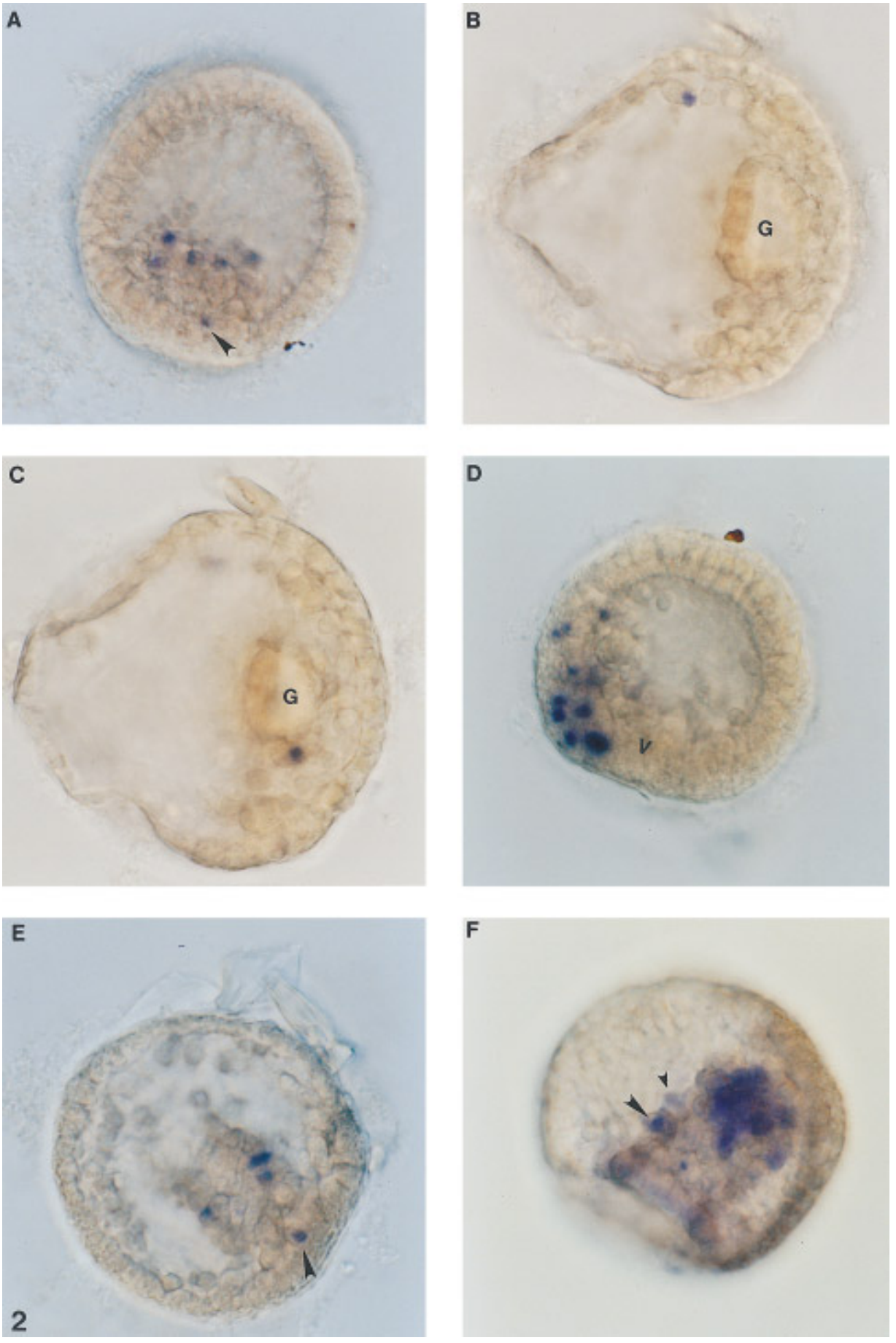
The 0.3CAT construct, unlike the endogenous *SM30*, is expressed at high levels beginning at about 24 hr. *In situ* hybridization analysis shows that this expression occurs in patches of cells in the vegetal plate (Fig. 2D) and PMCs (data not shown) of 24-hr blastulae. *In situ* hybridization analysis of 48-hr prism stage embryos harboring 0.3CAT show label in midgut (Fig. 2E) and foregut cells (Fig. 2F) as well as in mesenchyme cells (arrowheads, Figs. 2E and 2F) (Table 1). Since the gut is derived from the vegetal plate, the results at 48 and 24 hr are entirely consistent with one another. The number of vegetal plate and gut cells expressing 0.3CAT (shown in Fig. 2 and in Table 1) is similar to previous reports of the number of vegetal plate and gut cells expressing *Endo16* reporters in *S. purpuratus* (Yuh *et al.*, 1994). *Endo16* is a gut-specific marker. We conclude that 0.3CAT is correctly expressed in PMCs and inappropriately expressed in the vegetal-plate-derived gut territory.

The 2.6CAT, 1.6CAT, and 0.3CAT constructs show similar average numbers of mesenchyme cells expressing reporter per positive embryo, and there is no inherent difference in the efficiency of replication of any of the injected constructs, as observed by DNA dot blotting (data not shown). This rules out the possibility that the territorial difference in expression between 2.6CAT and 1.6CAT versus 0.3CAT is due to a fundamental difference in the degree of mosaicism obtained in embryos harboring these constructs. We conclude that the elements required for mesenchyme-specific spatial regulation of *SM30-α* exist within the −1628 to −300 region. Taken together with the analysis of CAT enzyme activity, the *in situ* results suggest that the 0.3CAT construct expresses more CAT enzyme than the 2.6CAT and 1.6CAT constructs because its domain of expression includes the vegetal-plate-derived territories.

Identification of Two Protein Binding Sequences within the Distal Repressive Region

DNase I protection experiments have been performed using crude nuclear extract and subclones of the upstream region from −1628 to −128 as templates. Two different sequence motifs, (G/A/C)CCCCT and (T/C)(T/A/C)CTTTT(T/A/C), were consistently protected in these experiments (Frudakis, 1995). Both motifs are numerous in the putative repressive region that spans −1628 to −300 (see Fig. 7). A fragment from −1478 to −1436 of the regulatory region (oligo(A), Fig. 3A), which contains representatives of both of these motifs, was selected for further study. Mutant and truncated versions of oligo(A) were synthesized to isolate each motif; oligo(C) contains an isolated GCCCCT sequence and oligo(D) contains isolated CTTTT sequences (Fig. 3A).

If the GCCCCT sequence is recognized by a sequence-specific nuclear protein, then we should observe a binding activity common to oligo(A) and oligo(C) but not oligo(D). Nuclear proteins were extracted, incubated with labeled oligo(A) and subjected to gel electrophoresis. Oligo(A) interacted with proteins in this nuclear extract (Fig. 3B, lane 1).


A
B
G
C
G
D
V
E
2
F

Several of the bands comprising the oligo(A) banding pattern are relatively refractory to competition by a 500× or 250× molar excess of unlabeled oligo(D) concatenates (Fig. 3B, lanes 2 and 3) but are susceptible to competition by a 500× or 250× molar excess of unlabeled oligo(C) concatenates (Fig. 3B, lanes 4 and 5). This competition is not observed when other nonspecific oligonucleotides are used, including those containing the *Eco*RI sequence (data not shown). Thus, the results indicate that these bands in the gel shift experiment using oligo(A) are the result of a specific interaction of nuclear proteins with some or all of the sequence TGACTCGTGCCCCTT (compare the sequence of oligo(A) and oligo(C), Fig. 3A). Additional gel mobility shift experiments using different restriction fragment subclones of the *SM30-α* upstream region as competitors only showed these characteristic mobility shifts when GCCCCT sequences were present in the restriction fragment (Frudakis, 1995).

Similar mobility shift experiments were carried out for the CTTTT sequence motif that is present at −1457 to −1436. Reproducible, but relatively weak, binding was observed for oligo(D), which contains two perfect and one imperfect CTTTT elements (data not shown). Since the (T/C)(T/A/C)CTTTT(T/A/C) motif is so highly repetitive in the *SM30-α* regulatory region (see oligo(A) sequence in Fig. 3A; Fig. 7), we tested the binding ability of an oligo(3D) concatenate, containing 9 CTTTT elements (Fig. 3A). Oligo(3D) interacts more strongly with proteins present in crude nuclear extract (Fig. 3C, lane 1) and forms the same banding pattern observed with oligo(D). A 500× molar excess of an unlabeled nonspecific competitor concatenate (oligo(C)) did not completely eliminate the bands (Fig. 3C, lane 2) but a 500× molar excess of unlabeled oligo(D)

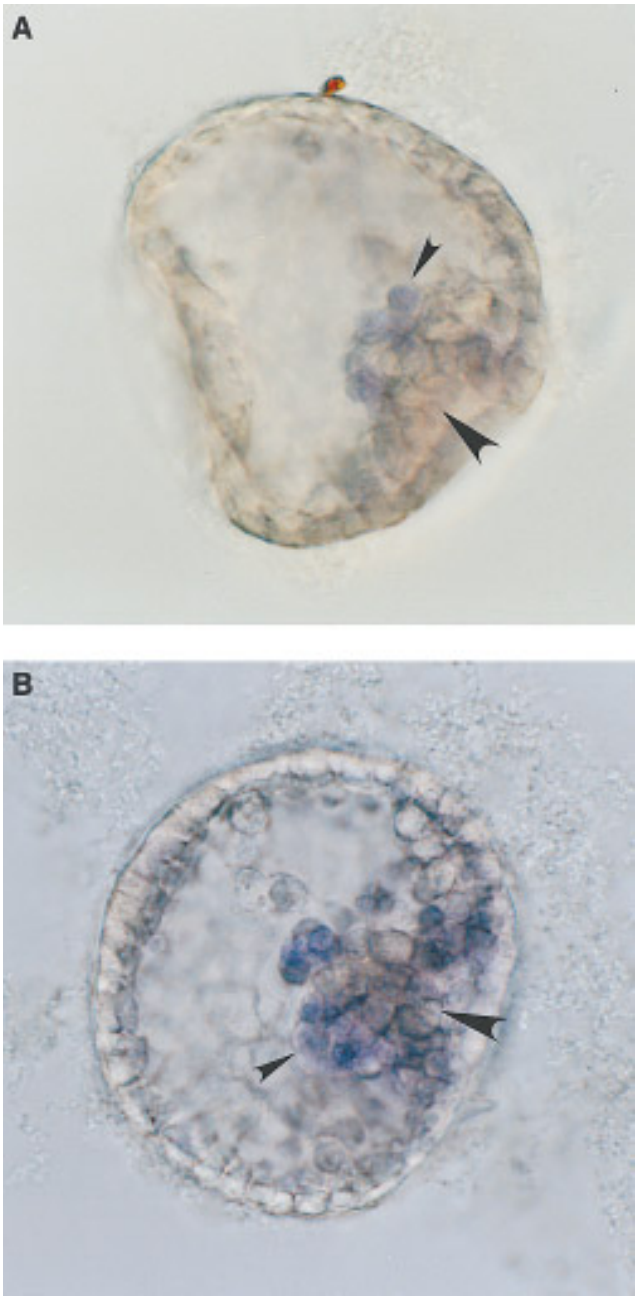


FIG. 2. Whole-mount *in situ* hybridization against the CAT message in *S. purpuratus* embryos harboring *SM30-α* deletion constructs. Positive cells are identified by purple color. Scale bar, 20 μm. (A) Lateral view of a 24-hr blastula harboring 1.6CAT, showing several positive PMCs inside the blastocoel and another, more posterior (arrowhead), that is in the process of ingression. This particular focal plane reveals the color from each of the positive cells in this embryo, although no one particular labeled cell is in precise focus. Nuclear staining may be an indication of weaker reporter activity in this individual. (B) View along the axis of the gut of a 48-hr gastrula harboring 1.6CAT. One positive cell is a visible part of the lateral chain of PMCs known as the lateral body-rod chain, which can be seen in this photograph wrapping around the gut (G) and extending to the left, above and below the gut. (C) More posterior focal plane of the same embryo shown in (B). A second stained PMC is visible immediately lateral to (below) the gut (G) and in the lateral PMC cluster. (D) Lateral view of a 24-hr mesenchyme blastula harboring the 0.3CAT construct. Several positive cells are seen in the left half of the vegetal plate (V). (E) Oral view of a 48-hr gastrula harboring 0.3CAT, showing three clusters of stained midgut cells. In this focal plane, a single stained PMC can be seen near the base of the gut (arrowhead). (F) Lateral view of a 48-hr gastrula harboring the 0.3CAT construct. Positive cells constitute a significant portion of the presumptive foregut. A stained mesenchyme cell is visible to the left of the gut (large arrowhead) and another exists out of the plane of focus below this cell (small arrowhead).

FIG. 5. Co-injection of oligo(C) or oligo(D) competitors with 2.6 CAT results in weak expression in the archenteron. Scale bar, 20 μm. (A) Thirty-hour gastrula harboring the 2.6CAT construct and oligo(C) competitor shows patches of weakly stained archenteron cells (small arrowhead) and patches of unstained archenteron cells (large arrowhead). (B) Thirty-hour gastrula harboring the 2.6 CAT construct and oligo(D) competitors shows patch of weakly stained archenteron cells (small arrowhead) adjacent to a region of unstained archenteron cells (large arrowhead).

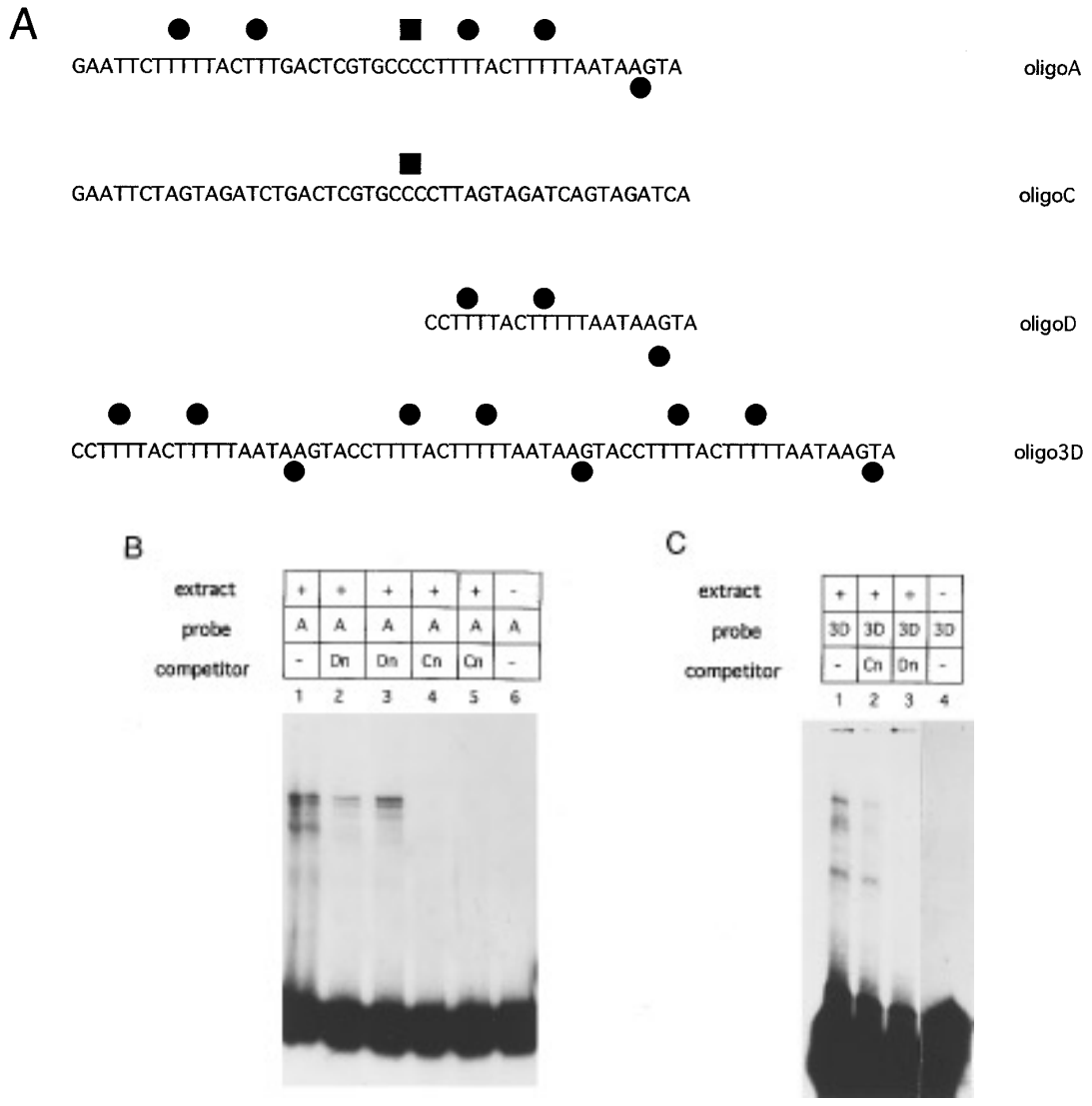


FIG. 3. Oligonucleotides containing GCCCCT and CTTTT sequences interact specifically with *S. purpuratus* nuclear proteins. (A) The sequences for oligonucleotides used in gel mobility shift assays. Oligo(A) is the sequence from −1478 to −1436 relative to the transcription start site (see Akasaka *et al.*, 1994). The oligos listed below oligo(A) are mutant or truncated versions of oligo(A). *Eco*RI linker sequence exists at the 5′ end of oligo(A) and oligo(C). The position of oligo(D) sequence relative to oligo(A) sequence shows that this oligo is the 3′ portion of oligo(A). Circles and squares above the oligo sequences denote CTTTT and GCCCCT sequence elements, respectively. (B) Gel mobility shift assay using labeled oligo(A) in crude nuclear extract showing that this oligo is bound by nuclear proteins in a sequence-specific manner. Competitors are present in 250-fold molar excess, except lanes 2 and 4, which contain 500-fold excess. An ''n'' subscript for a competitor signifies that this competitor was a concatenate of average 1-kb size. (C) Gel mobility shift assay using labeled oligo(C) in crude nuclear extract showing that this oligo is bound by nuclear proteins in a sequence-specific manner. A 500-fold excess of competitor was used. The notation is the same as described for (B).

concatenates did so (Fig. 3C, lane 3). Therefore, we conclude that there is sequence-specific binding to the CTTTT sequence at −1457 to −1436 by nuclear protein(s).

Relative affinity constants (K_r) for the interactions of nuclear proteins with sites residing in oligo(A) (see Fig. 3B) are estimated to be in the range from 10^5 to 10^7 (data not shown; see Materials and Methods), indicating that binding specificity is probably high. We therefore wished to evaluate the possible function of these two elements in reporter construct regulation.

Assessing Sequence Function with in Vivo Competition

One approach to the function(s) of *cis* elements in regulation of reporter construct expression is to co-inject with

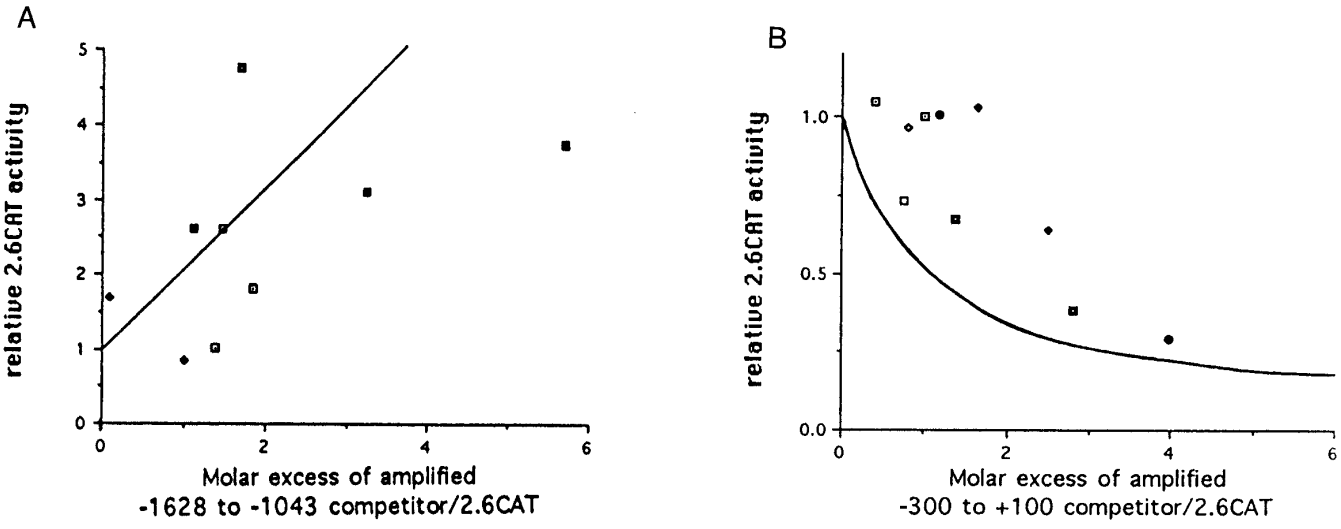


FIG. 4. *In vivo* competition experiments show that the factors driving expression of the 2.6CAT construct can be titrated by the coinjection of competitor fragments containing *SM30-α* regulatory sequence. (A) Co-injection and amplification of a competitor spanning from −1628 to −1043 of the *SM30α* regulatory region along with 2.6CAT reporter results in an increase in 2.6CAT activity in 48-hr embryos. Shown is a plot of the change in CAT activity as a function of the ratio of amplified competitor to reporter sequences. The change in CAT activity effected by the competitor sequences is found by first determining the standardized CAT activity (see Materials and Methods) from batches of embryos injected with 2.6CAT and 5- to 10-fold molar excess of the competitor. The activity from these batches is then divided by the standardized CAT activity of a control batch of embryos injected with 2.6CAT and 5- to 10-fold molar excess of a nonspecific (bluescript plasmid) restriction fragment to give a relative CAT activity quotient. The ratio of competitor to reporter sequences that have been amplified for each batch of injected embryos is determined by calculating the number of amplified competitor sequences for the batch and dividing this number by the number of amplified 2.6CAT reporter sequences for the batch. The relative CAT activity quotient is then plotted against the ratio of competitor to reporter sequences. The line shown is the theoretical first order change in 2.6CAT activity expected from a stoichiometric titration of negative regulatory factors from the 2.6CAT reporter, given by the function $f(X) = 1 + X$, where X is the molar ratio of amplified competitor to 2.6CAT reporter. (B) Plot of 2.6CAT activity under the competitive influence of a fragment spanning the −300 to +105 region of the *SM30-α* promoter as in (A). The line represents the change in reporter activity expected from a stoichiometric titration of positive regulatory factors given by the function $f(X) = 1/(1 = X)$, where X is the molar ratio of amplified competitor/reporter sequences (Livant *et al.*, 1988).

the reporter an unlinked polynucleotide with the same, or similar, sequence as the suspect *cis* regulatory element (Livant *et al.*, 1988). The unlinked "competitor" sequence may then compete for DNA binding proteins which may be involved in regulation. This approach was useful in dissecting the regulation of the *CyIIIa* gene of sea urchins (Franks *et al.*, 1990; Hough-Evans *et al.*, 1990).

Figure 4A shows the results of co-injection of a 585-bp fragment spanning −1628 to −1043 together with the 2.6CAT reporter. This region contains mainly GCCCCT and CTTTT elements (Fig. 7). Even though a 5- to 10-fold molar excess of competitor was co-injected, measurements of competitor/reporter after embryonic development show a much lower relative degree of amplification of competitor. There is scatter in the data, especially at lower values of competitor/reporter, but in seven of nine experiments co-injection of the putative competitor stimulated CAT enzyme activity compared to controls, and in instances in which higher values of competitor/reporter were obtained the stimulation was substantial. This is consistent with the region from −1.6 to −1.0 kb subserving a negative regulatory role.

When the 2.6CAT construct is challenged with a 405-bp fragment spanning −300 to +105, the result is very different (Fig. 5B). The −300 to +105 region, as shown in the deletion analysis, apparently contains positive regulatory elements. At low competitor/reporter ratios there is scatter in the data, but stimulation of CAT activity was never observed. In 5 of 10 experiments the CAT activity is depressed, and at higher ratios of competitor/reporter the depression of enzyme activity is marked. This is consistent with the idea that this region subserves positive regulatory functions and by contrast serves as a control on the effects obtained with the −1628 to −1043 region.

Our attempts to exploit this approach in more detail by using smaller restriction fragments and oligonucleotides was confounded by the scatter in the data that could be obtained and the approach was not pursued further. However, we did obtain important additional information by subjecting embryos that had received co-injected competitors to *in situ* hybridization. Figure 5A shows an example of staining of an embryo injected with 2.6CAT together with oligo(C) (containing GCCCCT). There is faint but clearly visible expression of CAT in cells of the invaginating

archenteron. Figure 5B indicates a similar result after co-injection of oligo(D) (containing CTTTT) with 2.6CAT. 2.6CAT alone is never expressed in prospective or differentiated gut cells (Akasaka *et al.*, 1994). Co-injection of a 43-bp fragment from pBluescript (*Pst*I to *Xho*I) produced no effect whatsoever on the expression of 2.6CAT in PMCs, and co-injection of the −300 to +105 region (see Fig. 4B) reduced the expression of 2.6CAT below the threshold that could be observed by the procedures used. We are not certain why the expression of 2.6CAT in archenteron cells caused by co-injection of oligo(C) or oligo(D) is weak; the difficulty that we experienced in obtaining high levels of competitor/reporter in competition experiments may be contributory. Nonetheless, the indications are clear that oligo(C) and oligo(D) may be involved in negative control elements that prevent expression of 2.6CAT in the developing gut.

Assessing Sequence Function with Fusion Constructs

If the proteins interacting with the upstream GCCCCT and CTTTT sequences in the cell are negative regulators, and if these regulators are competent to function outside of their natural sequence environment, then fusion of oligonucleotides containing these elements upstream to the −300 to +100 regulatory region would be expected to result in a decrease of the otherwise robust CAT activity shown by reporters containing only the −300 to +105 region. Fusion constructs were created by inserting either oligo(C) or oligo(D) sequences upstream of the *SM30-α* promoter sequence in 0.3CAT (Fig. 6A), microinjecting CAT into fertilized zygotes, and assaying for CAT enzyme activity and fusion construct replication. The results show that neither oligo(C) nor oligo(D) multimers show an ability to suppress 0.3CAT activity (Fig. 6B). In fact, oligo(C) enhances 0.3CAT activity, suggesting that the TGACTCGTGCCCCTT sequence (the only nonrandom sequence in oligo(C)), may function independently as an activator of transcription.

However, the TGACTGGTGCCCCTT and CTTTT sequences at −1478 to −1436 flank one another (see oligo (A), Fig. 3A). In fact, 14/22 of the (G/A/T)CCCCT motifs in the −1628 to −300 domain of the regulatory region are immediately flanked by a (T/C)(T/A/C)CTTTT(T/A/C) motif (see Fig. 7; for sequence, see Akasaka *et al.*, 1994). Thus, we tested the function of this tandem sequence arrangement by fusing oligo(A) multimers upstream of the 0.3CAT construct (Fig. 6A). Figure 6B shows that one, two, or five copies of oligo(A) substantially depress 0.3CAT activity in microinjected embryos. The results suggest that neither synthetic oligonucleotide (C) or oligo(D), containing isolated GCCCCT or CTTTT sequences, respectively, is sufficient to effect substantial transcriptional repression upon the 0.3CAT construct; only the natural sequence arrangement at −1478 to −1436, containing both elements immediately juxtaposed, can repress transcription driven by the −300 to +105 region.

To assess where in the embryo this composite repressive element functions, whole-mount *in situ* hybridization was used. Table 2 shows the tabulated results from five experiments; oligonucleotides containing isolated GCCCCT or CTTTT sequences did not influence expression of the 0.3CAT construct in gut cells. However, oligo(A), which contains both of these sequences, caused a significant reduction in the incidence of 0.3CAT expression in the gut. This result suggests that the natural oligonucleotide sequence at −1478 to −1436, which contains both GCCCCT and CTTTT sequence, is sufficient to negatively regulate transcription driven by the −300 to +105 region in this territory.

TABLE 2

In Situ Hybridization Experiments for 48-hr Embryos Harboring 0.3CAT Fusion Constructs

Construct	Number of positive embryos			Approximate no. embryos injected
	Ectoderm	Gut	Mesenchyme	
0.3CAT	2	88	99	1000
5A0.3CAT	1	8	85	1000
2C0.3CAT	3	51	104	1000
5D0.3CAT	3	30	40	600

DISCUSSION

Our results show that the *SM30-α* DNA from −1648 to +105 is necessary and sufficient to confer a mesenchyme-specific pattern of reporter gene transcription in *S. purpuratus*. In contrast, the sequence from −300 to +105 directs expression of reporter genes in the vegetal-plate-derived gut territory as well as in mesenchyme. The proximal −300 to +105 region must contain a preponderance of positive elements for *SM30α* expression. The restraint of *SM30α* expression in ectoderm may also be due in part to mechanisms that involve this region, or molecules that reside in ectoderm cells, or both. This rather complex matter is the subject of ongoing investigation.

Our observations suggest that the primary mesenchyme-specific pattern of *SM30-α* expression results, at least in part, from an active process of negative spatial regulation imposed by distal elements upon a proximal promoter. This may be a common tactic in the development of many embryos. We have shown that elements required for repression in the gut territory reside within the −1628 to −300 domain of the regulatory region. This domain contains multiple copies of two distinct sequence motifs (GCCCCT-like and CTTTT-like sequences) that exhibit DNase I protection, and oligonucleotides containing representatives of these motifs at −1478 to −1436 were isolated and found to bind nuclear proteins in a sequence-specific manner. The results suggest that both of these sequences at −1478 to −1436 are *required* for proper repression of the 2.6CAT construct in the gut, but only a tandem arrangement of these two se-

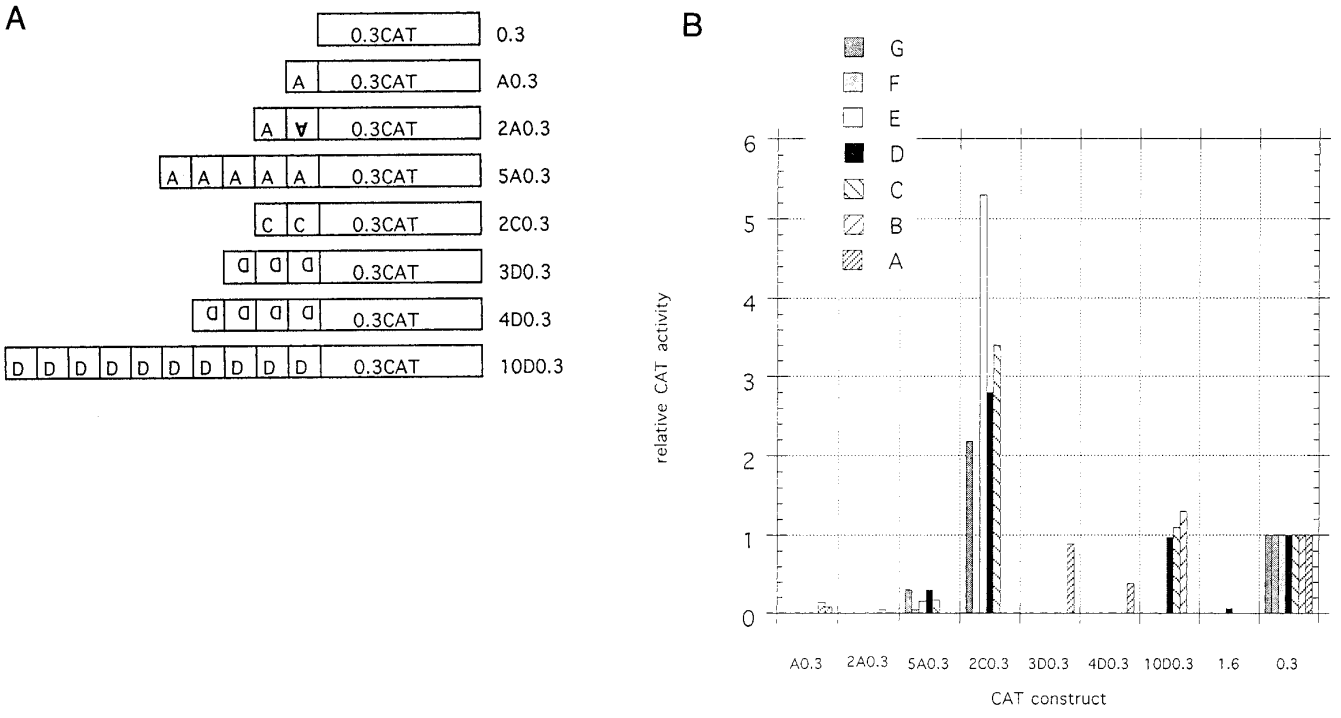


FIG. 6. Only oligonucleotides containing both a GCCCCT and CTTTT sequence element confer repression upon the promiscuous 0.3CAT reporter construct. (A) Schematic depiction of oligonucleotide fusion constructs. The letters A, C, and D correspond to the oligonucleotides of Fig. 5. Reverse letters signify that the orientation of the oligo is inverted from the 5′ to 3′ orientation shown in Fig. 5A. (B) Histogram showing the relative CAT activity of the fusion constructs shown in (A). CAT activity is standardized against reporter amplification as described under Materials and Methods. The blocks at the top of the histogram denote different experiments. The absence of a bar for a given construct, in a given experiment, signifies that the construct was not among those assayed in that experiment. CAT activity for each construct is shown relative to that of the 0.3CAT construct, which is arbitrarily set at 1 for each experiment.

quences is *sufficient* to effect this repression. The combined results suggest that the repression of *SM30-α* reporters in the gut depends on the formation of a structure whose activity or binding requires both GCCCCT and CTTTT sequences.

The bipartite sequence at −1478 to −1436 is one of many similar composite elements distributed throughout the −1628 to −300 region. Figure 7 shows a diagram of the occurrence of both the (G/A/T)CCCCT and (T/C)(T/A/C)CTTTT(T/A/C) motifs (within one mismatch) in the 2.6-kb regulatory region. It is apparent from this diagram that the two motifs often occur coincidently; between −1628 and −300, 14 of 22 (G/A/T)CCCCT motifs are immediately flanked by a (T/C)(T/A/C)CTTTT(T/A/C) motif. In light of the results presented here, which implicate this bipartite sequence arrangement as a negative spatial control element, this coincident sequence distribution is probably significant. Consistent with this idea, the deletion construct results from this work suggest that repressive elements are redundantly distributed within the −1628 to −300 region. While imperfect representatives of both elements appear in the region from −300 to +105 (two GCCCCT and 5 CTTTT), the one tandem bipartite pair in this region has a more distant spacing of the two elements. It is worth noting that 34 of 39 (T/C)(T/A/C)CTTTT(T/A/C) motifs between −1628 and −1043 appear on the same strand of DNA (see Fig. 7).

The (T/C)(T/A/C)CTTTT(T/A/C) motif resembles three protein binding elements found in the *Endo16* regulatory region whose functions have not yet been defined nor have cDNAs encoding proteins binding to it yet been cloned (sites 5, 6, and 17; Yuh *et al.*, 1994). The (G/A/T)CCCCT motif is similar to the canonical binding site for the *S. purpuratus* Sp(G/C)F-1 protein (formerly known as P8; Theze *et al.*, 1990; Thiebaud *et al.*, 1990). In fact, the representative of this (G/A/T)CCCCT motif at −1456 of *SM30-α* binds avidly to the Sp(G/C)F-1 protein *in vitro* (Frudakis, 1995). Since Sp(G/C)F-1 interaction sites have been shown to function as activators in the context of the *CyIIIa* regulatory region (Franks *et al.*, 1990), it is interesting that an oligonucleotide containing this GCCCCT sequence at −1456, isolated from its natural sequence environment (oligo (C)), enhances the expression driven by the −300 to +105 region of *SM30-α* (see Fig. 6).

Several other sea urchin genes are known to be regulated by modular cassettes of repetitive sequence elements (Gan

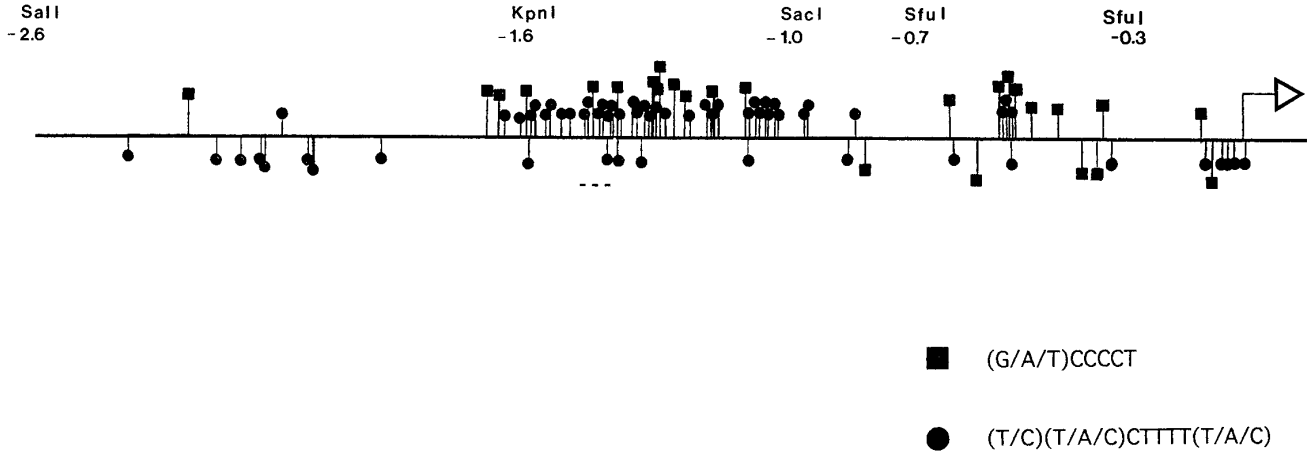


FIG. 7. Schematic depiction of the *SM30-α* 2.6-kb regulatory region showing the occurrence of sequences matching the degenerate (G/A/C)CCCCT (square pins) and (T/C)(T/A/C)CTTTT(T/A/C) (circle pins) sequences with no more than one mismatch. For sequence, see Akasaka *et al.* (1994). All of the sequences noted with a square contain a core CCCC sequence and those noted with a circle contain a core CTTTT sequence. The distance from the start site in Kilobases and the corresponding restriction sites are shown above the diagram. The region from which the oligonucleotides (described in the text and shown in Fig. 3A) were isolated is shown with a dashed line. Pins above the line indicate that the sequence element appears on the sense strand and those below indicate that the sequence element appears on the antisense strand.

and Klein, 1990; Nemer *et al.*, 1993; Anderson *et al.*, 1994). The bipartite (G/A/C)CCCCT and (T/C)(T/A/C)CTTTT(C/T) motif-rich regions in the *SM30-α* upstream region may meet the criteria defining such a cassette (reviewed in Dynan, 1989). If this is so, then regions rich with these two sequences will be found to be functionally important in other promoters. Sequences within one mismatch of the (G/A/T)CCCCT and (T/C)(T/A/C)CTTTT(T/A/C) motifs are repetitively and coincidently distributed in the upstream region of the PMC-specific *PM27* gene (at −67 to −86, −288 to −390, and −698 to −718; for sequence see Raman *et al.*, 1993) and PMC-specific *msp130* gene (at −177 to −165, −232 to −267, −381 to −404, and −475 to −486; for sequence see Parr *et al.*, 1990). Furthermore, deletion of sequence spanning the four (G/A/T)CCCCT and (T/C)(T/A/C)CTTTT(T/A/C) motif-containing regions in the *msp130* upstream region results in expression of a reporter in gut cells (K. Klueg and R. Raff, personal communication). These elements are also found together, on the same strand of DNA, in the regulatory region of the gut-specific *Endo16* gene (nuclear protein interaction site 17; Yuh *et al.*, 1994). It will be interesting to test the idea in future work that coincident (G/A/T)CCCCT and (T/C)(T/A/C)CTTTT(T/A/C) sequences function as a regulatory module in the transcriptional regulation of embryonic gene expression in *S. purpuratus*.

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